Stimulation of Photorespiration by the Carbonic Anhydrase Inhibitor Ethoxyzolamide in *Chlorella vulgaris*

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Dedicated to Prof. Dr. Dr. Josef Straub at the occasion of his 75th birthday

Ammonia Excretion, Glycolate Excretion, CO_2 Concentration, *Chlorella vulgaris,* L-MSO Treatment, Photorespiratory Nitrogen Cycle

Ammonia was excreted at high rates in the presence of L-methionine sulfoximine (L-MSO) from *Chlorella* cells which have been grown and analyzed at normal CO_2 partial pressure (330 ppm). If these cells are analyzed at high CO_2-concentration (3% CO_2 in air) only little ammonia is excreted in the presence of L-MSO. In the absence of L-MSO no ammonia is excreted under either condition. In agreement with this observation *Chlorella* cells *grown* under high CO_2 partial pressure (3% CO_2 in air) but tested under normal CO_2 partial pressure excreted only very little ammonia. Under these conditions neither "High CO_2-cells" nor "Low CO_2-cells" exhibited any glycolate excretion. However, glycolate excretion was observed in the presence of α-HPMS (α-hydroxy-2-pyridyl methanesulfonate) an inhibitor of glycolate dehydrogenase or INH (isonicotinyl hydrazide) an inhibitor of the glycine-serine aminotransferase, irrespective of the presence or absence of L-MSO. INH inhibited ammonia excretion. The above described high ammonia excretion in "Low CO_2-cells" in the presence of L-MSO was suppressed or substantially reduced by 0.1 mM ethoxyzolamide an inhibitor of carbonic anhydrase which, however, at the same time caused a substantial excretion of glycolate into the medium. The same qualitative effect of ethoxyzolamide was observed in "High CO_2-cells" (tested under normal CO_2 partial pressure) although the amount of glycolate excreted in this type of culture was very small. It was generally noted that glycolate excretion caused by ethoxyzolamide was stoichiometrically always more important than the rate of ammonia excretion which was inhibited. This shows that excretion and therefore most probably also the formation of glycolate are enhanced by ethoxyzolamide. The experiments seem to show that due to the inhibition of carbonic anhydrase the affinity of the ribulose-1,5-bisphosphate carboxylase/oxygenase system is increased towards oxygen, which leads to a stimulation of the photorespiratory carbon cycle.

Introduction

Since the time when Tolbert and Zill [1] first reported that *High CO_2-cells* of *Chlorella* excreted glycolate into the culture medium under high light intensity and high O_2 tension, many laboratories have reported on glycolate formation and excretion in different algae [2–7]. Using higher plants, Key *et al.* [8] reported on the photorespiratory nitrogen cycle and its relationship to the glycolate pathway. Thus, in the glycolate pathway, two molecules of glycine are converted to one molecule of serine, CO_2 and ammonia in the mitochondria. The ammonia released from mitochondria is refixed by the enzyme glutamine synthetase which is located in the chloroplast [9]. When the reaction of glutamine synthetase is inhibited by L-MSO, photosynthesis also appears to be inhibited which leads to the consequence that a great amount of ammonia is accumulated in the cells [10]. This clearly shows that the photorespiratory nitrogen cycle, namely refixation of ammonia by glutamine synthetase, plays an important role in photosynthesis.

On the other hand, it has been reported that many algal cells grown under high CO_2 partial pressure (~ 2–3%) have a lower affinity for CO_2 in photosynthesis than those grown under low CO_2 which is the normal CO_2 concentration (330 ppm) [11–15]. This has led to the notion that the activity of carbonic anhydrase in many algae is higher in *Low CO_2-cells* than that in *High CO_2-cells*. It was concluded for

Abbreviations: L-MSO, L-methionine-DL-sulfoximine; ethoxyzolamide, 6-ethoxybenzothiazole-2-sulfonamide; α-HPMS, α-hydroxy-2-pyridyl methanesulfonate; INH, isonicotinyl hydrazide; *Low CO_2-cells,* algal cells grown in ordinary air, *i.e.* with 330 ppm CO_2; *High CO_2-cells,* algal cells grown in air supplemented with 3% CO_2.

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Chlorella vulgaris 211-11h that the high affinity for CO_2 in photosynthesis in *Low CO_2-cells* is due to the activity of carbonic anhydrase induced at low CO_2 concentration in the cells [16, 17]. For *Anabaena* and *Cocochloris* it was demonstrated that the observed high affinity for CO_2 is due to an active bicarbonate accumulation mechanism, induced in *Low CO_2-cells* [18, 19]. As Tsuzuki and Miyachi [20] have reported, *High CO_2-cells* of *Chlorella vulgaris* 211-11h cells have a higher CO_2 compensation point and a more active photorespiration than *Low CO_2-cells*. These results demonstrate that glycolate formation and the photorespiratory nitrogen cycle are more active under CO_2-limiting conditions in *High CO_2-cells* rather than in *Low CO_2-cells*. This conclusion was confirmed by Colman *et al.* [5] who showed that glycolate excretion under CO_2 limiting conditions was more active in *High CO_2-cells* than in *Low CO_2-cells* of *Chlorella pyrenoidosa*.

In the present paper we show that glycolate and ammonia excretion follow a different pattern in *Chlorella vulgaris* 211−11 h.

Materials and Methods

Algae: *Chlorella vulgaris* 211-11h originates from the algal collection of the University of Göttingen; an alternative name is *C. kessleri* [21]. The alga was the generous gift of Prof. W. Kowallik, Bielefeld. The algae were grown under continuous illumination in a 250-ml flask containing ammonia-free inorganic medium, as described previously [15, 22]. The algal suspension was continuously bubbled with air containing 3% CO_2. After a few days, the algal suspension was divided into two samples. One was kept under the same conditions *(High CO_2-cells)* while the gas bubbled into the other sample was changed to ordinary air containing 0.03% CO_2 *(Low CO_2-cells)*. The temperature was kept constant at 25 °C. The algal cells were harvested by centrifugation (3,000 rpm for 5 min) and were suspended in 100 ml of 25 or 50 mM MES-NaOH buffer, pH 6.0, containing an ammonia-free culture medium (5% of total volume). All algal suspensions were kept under identical conditions which is also valid for those with the addition of L-MSO (final concentration, 0.5 mM). The algal cultures were illuminated by fluorescent lamps (10 klux) and kept at 25 °C. For the analysis, at the chosen time intervals the algal suspension was centrifuged 5 min at 3500 rpm at 4 °C with the supernatant used for the glycolate or ammonia determination.

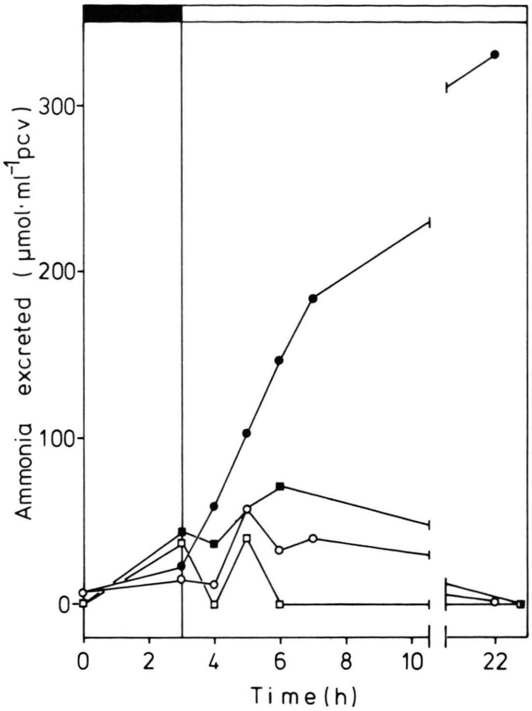
Concentrations of glycolate and ammonia were determined colorimetrically using the methods of Calkins [23] and Weatherburn [24], respectively. Other conditions are given when necessary in the figure legends.

Chemicals used were L-MSO, L-methionine-DL-sulfoximine, INH, isonicotinic acid hydrazide and α-HPMS, α-hydroxy-2-pyridyl methanesulfonate which were purchased from Aldrich Co. Ltd. Ethoxyzolamide, 6-ethoxybenzothiazole-2-sulfonamide was the gift of Dr. Thilo and Co. GmbH, Sauerlach, Munich, Germany.

Results

If *High CO_2-cells* are compared to *Low CO_2-cells*, both analyzed under normal CO_2 partial pressure (330 ppm CO_2), it is clearly seen that only in the presence of L-MSO ammonia excretion is observed. This excretion is substantially higher in *Low CO_2-cells* (Fig. 1a, b). If the cells are analyzed under high CO_2 partial pressure, 3% CO_2 that is, no ammonia is excreted. In the absence of L-MSO neither under high nor low CO_2 partial pressure ammonia excretion is observed (Fig. 1). If *Low CO_2-cells* are transferred to 3% CO_2, ammonia excretion is inhibited whereas glycolate excretion remains unchanged low (Fig. 2). Isonicotinyl hydrazide (INH) which is an inhibitor of glycine-serineaminotransferase [25] clearly inhibits ammonia excretion in *High* and *Low CO_2-cells*, which is expected according to the literature (Fig. 3). At the same time the addition of INH causes in both types of cells a substantial glycolate excretion which is also in agreement with the literature [5]. L-MSO does not interfer with the effect of INH on glycolate excretion with the restriction that absence of L-MSO apparently causes a lag period for glycolate excretion (Fig. 3b). If ethoxyzolamide an inhibitor of carbonic anhydrase is added to the experimental system of Fig. 1 in which ammonia excretion is observed, a clear-cut inhibition of this ammonia excretion is observed (Fig. 4). Ammonia excretion of *Low CO_2-cells* in the presence of L-MSO is lowered to the level of *High CO_2-cells*. By the same time at which ammonia excretion is inhibited, a substantial glycolate excretion starts in *Low CO_2-cells* (Fig. 5a) as well as

1a



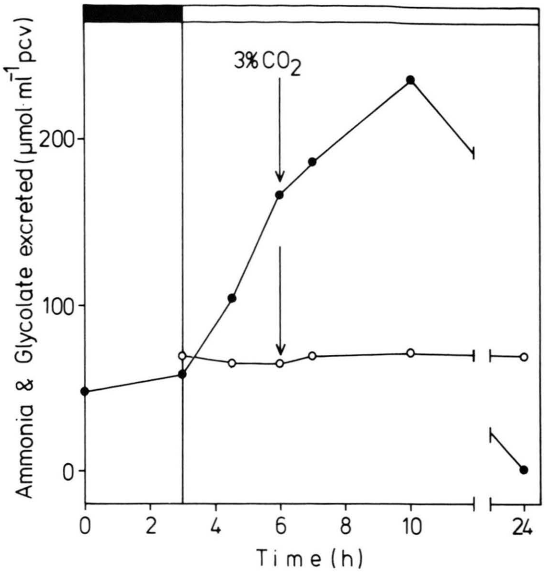


Fig. 2. Effect of CO_2 concentration on ammonia (●) and glycolate (○) excretion in the presence of L-MSO in *Low CO_2-cells* of *Chlorella vulgaris* 211−11h. The arrow indicates the change of CO_2 concentration from 0.03% to 3%. The CO_2 concentrations were set up by bubbling the algal cultures constantly.

1b

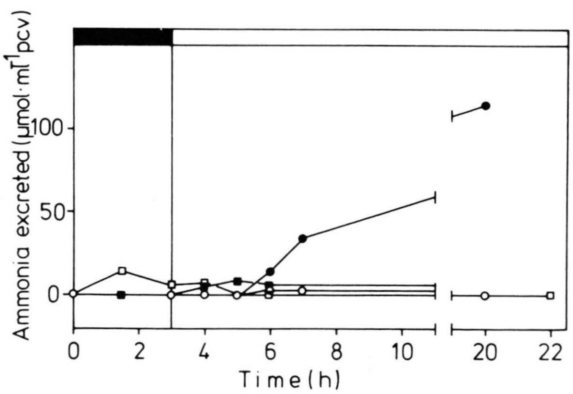


Fig. 1. Time course of ammonia excretion in the presence (●) and absence (○) of L-MSO under 0.03% CO_2 in air in the presence (■) and absence (□) of L-MSO in 3% CO_2 in air. a) *Low CO_2-cells* and b) *High CO_2-cells*. L-MSO (L-methionine sulfoximin) was applied as described in Materials and Methods. As in the following figures the black and white bar indicates dark and light period respectively. Moreover, as valid for this figure and all following ones the CO_2 concentration was set up by gassing constantly with either 0.03% CO_2 in air or with 3% CO_2 in air.

in *High CO_2-cells*. It looks as if ethoxyzolamide affected besides the enzyme carbonic anhydrase also the glycolate/glyoxylate oxidoreductase. This, however, is not conclusive as glycolate excretion, does not match stoichiometrically the ammonia excretion from before. It rather looks as if glycolate excretion exceeded considerably ammonia excretion which would speak in favour of an enhancement of photorespiration measured as glycolate production. If the latter argument fits, blocking of the glycolate-glyoxylate transition by α-hydroxy-2-pyridyl methane sulfonate (α-HPMS) according to Zelitch [26] should lead to glycolate excretion which in turn should be enhanced upon addition of ethoxyzolamide. Fig. 5c shows that this is indeed the case. Thus, the interpretation of our phenomenon should be that ethoxyzolamide by inhibition of carbonic anhydrase increases the affinity of the ribulose-1,5-bisphosphate carboxylase oxygenase system for oxygen which thus leads to an enhancement of photorespiration which we measure as glycolate excretion. If this was true photorespiration measured as $^{18}O_2$-uptake should be considerably enhanced by ethoxyzolamide. Preliminary results indicate that this is the case (the mass spectrometric analysis is in preparation).

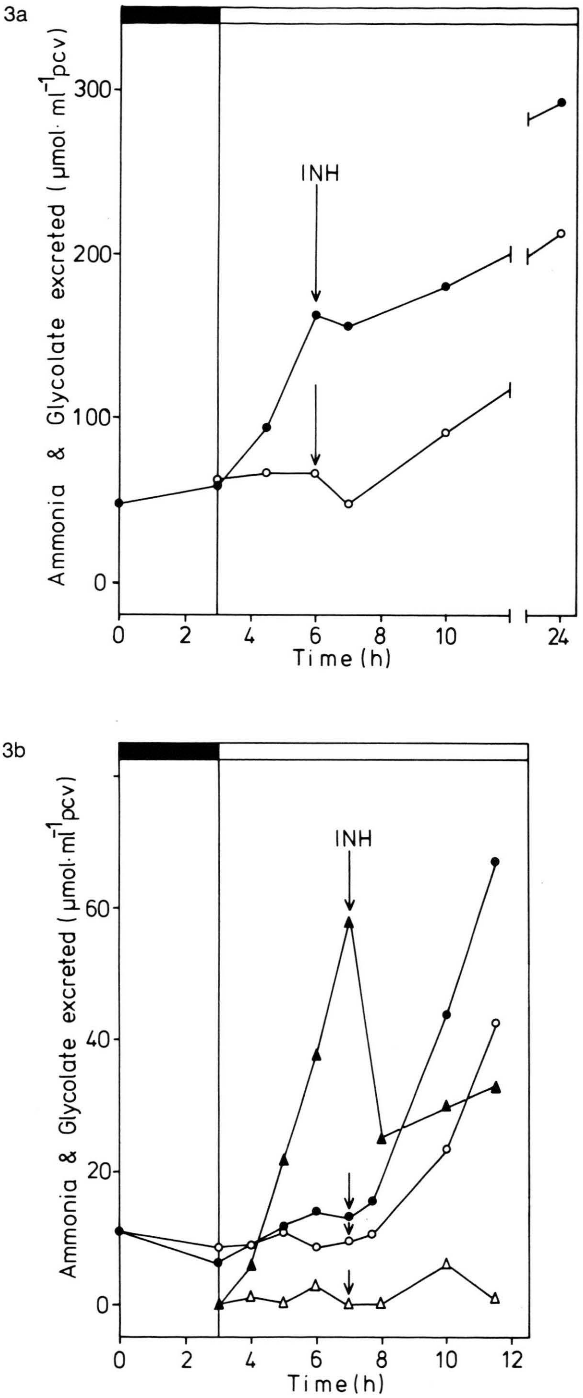


Fig. 3. a) Effect of isonicotinyl hydrazide (INH) on ammonia (●) and glycolate (○) excretion in the presence of L-MSO in *Low CO_2-cells.*
b) *High CO_2-cells:* ammonia excretion in the presence (▲) and absence (△) of L-MSO; glycolate excretion in the presence (●) and absence (○) of L-MSO.

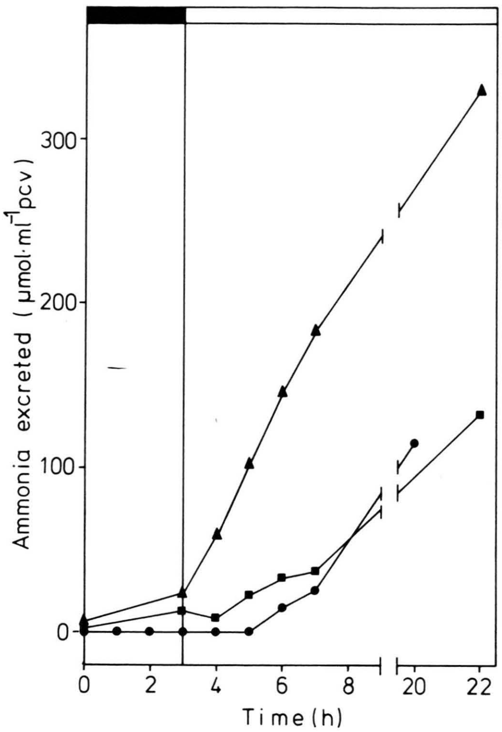
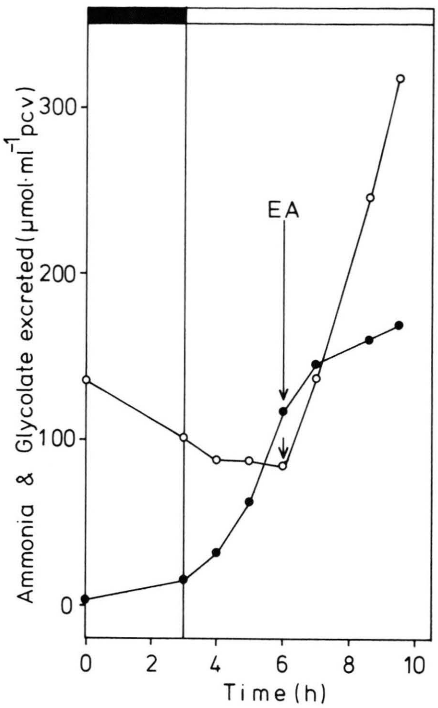


Fig. 4. Time course of ammonia excretion in the presence of L-MSO in *Low CO_2-cells* (▲), in *High CO_2-cells* (●) and in *Low CO_2-cells* which have been treated with ethoxyzolamide (EA) (■).

Discussion

Keys *et al.* have described that photorespiration finds its role in the nitrogen metabolism of higher plants [8]. Thus, the ammonia released from the glycolate pathway during photorespiration is recycled or refixed by the action of glutamine synthetase in chloroplasts. Somerville and Ogren have confirmed this aspect by using photorespiratory mutants of *Arabidopsis* [27]. Very recently Peltier and Thibault [10] were able to show that ammonia excretion was observed in *Chlamydomonas* when glutamine synthetase was blocked by L-MSO, provided the cells were grown at low CO_2 concentrations. Moreover, the excretion responded to the CO_2 and O_2 partial pressure in the assay [10]. In the present paper we show that the activity of carbonic anhydrase affects photorespiratory activity measured as ammonia excretion (Fig. 1 a). According to the literature, carbonic anhydrase activity is induced in *Chlorella,* if the algae are grown

5a



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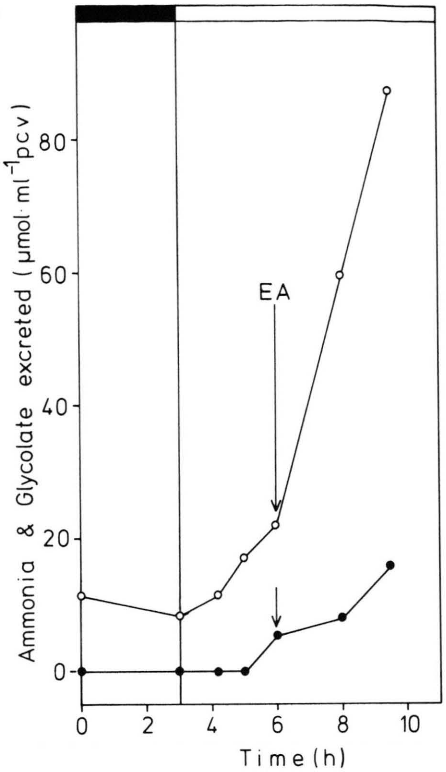

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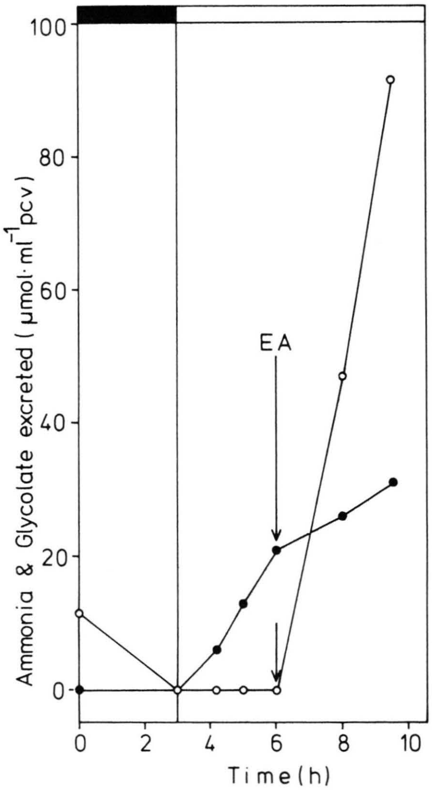

Fig. 5. a) *Low CO_2-cells* analyzed in 0.03% CO_2 in air: Ammonia (●) and glycolate (○) excretion in the presence of L-MSO. The arrow indicated the addition of ethoxyzolamide (EA).
b) *High CO_2-cells* analyzed in 0.03% CO_2 in air. Time course of ammonia (●) and glycolate (○) excretion in the presence of L-MSO. The arrow indicates addition of ethoxyzolamide.
c) *High CO_2-cells* analysed in 0.03% CO_2: Time course of ammonia (●) and glycolate (○) excretion in the presence of L-MSO plus α-HPMS. The arrow indicates addition of ethoxyzolamide (EA).

under low CO_2 partial pressure (*e.g.* 330 ppm) [15, 28]. If grown under 3% CO_2 the synthesis of carbonic anhydrase is suppressed [15, 28]. Common interpretation is that in the presence of a given carbonic anhydrase activity, a certain CO_2/O_2 ratio is available at the site of action of the enzyme ribulose-1,5-bisphosphosphate carboxylase/oxygenase permitting the corresponding activity of the carboxylating and oxygenase reaction. The oxygenase reaction metabolically supplies the glycolate pathway. Addition of L-MSO to *Low CO_2-cells* under our conditions (Fig. 1) leads to a substantial ammonia excretion which is in agreement with what has been exposed above [10]. The fact that the CO_2/O_2 ratio at the site of action of the carboxylase/oxygenase system is responsible for this ammonia excretion is demonstrated by the fact that further addition of CO_2, *i.e.* 3% completely inhibits this excretion (Fig. 1a). In this case carbonic anhydrase increases at the binding site of the enzyme the CO_2 concentration to the level at which the oxygenase function is virtually suppressed. It should be noted that under this condition when high CO_2 concentration suppresses ammonia excretion practically no glycolate excretion is observed (Fig. 2). If one lowers under these conditions the CO_2 concentration at the site of the carboxylase/oxygenase system by inhibiting carbonic anhydrase with ethoxyzolamide, ammonia excretion is inhibited instead of being enhanced as expected (Fig. 4). A closer scrutiny of the phenomenon reveals that the inhibition of ammonia excretion coincides with very active glycolate excretion (Fig. 5a). This experiment clearly shows that lowering the CO_2/O_2 ratio at the ribulose-1,5-bisphosphate carboxylase/oxygenase system leads to an enhanced photorespiratory activity which manifests itself as glycolate excretion: *Ethoxyzolamide seems to enhance photorespiration by enhancing glycolate production.* Stoichiometrically the enhanced glycolate production seems to exceed considerably the rate of ammonia excretion observed before the inhibition by ethoxyzolamide. In addition, the fact that ethoxyzolamide leads to an enhanced glycolate production and an inhibition of ammonia excretion might mean that ethoxyzolamide besides it inhibitory action on carbonic anhydrase also affects the glycolate/glyoxylate oxidoreductase or changes the permeability for glycolate. In a control experiment we induced glycolate excretion in our cells by α-HPMS according to Zelitch [26]. This excretion is caused by blocking the conversion of glycolate to glyoxylate. Addition of ethoxyzolamide to this condition clearly shows that lowering of the CO_2 concentration at the site of CO_2 fixation leads to an enhanced glycolate production (Fig. 5c).

What has been said to this point refers to *Low CO_2 Chlorella cells* which are cells grown under normal CO_2 partial pressure (0.03%). In this case our results are easily brought in line with those of the literature [5, 6]. However, under our conditions *High CO_2-cells i.e.* cells grown in 3% CO_2 and containing no carbonic anhydrase [16, 28] do not behave as expected from the literature. It has been reported that *High CO_2-cells* of *Coccochloris, Chlorella pyrenoidosa* and *Chlamydomonas* excrete more glycolate than *Low CO_2-cells.* In our case *High CO_2-cells* tested under normal CO_2 content (330 ppm) do not exhibit neither much photosynthetic nor much photorespiratory activity. This seems nearly trivial since these cells are not supposed to have carbonic anhydrase which means that the CO_2 concentration at the site of carboxylation is certainly limiting. Since CO_2 fixation with the production of ribulose-1,5-bisphosphate is low, photorespiration *e.g.* glycolate production or ammonia excretion are also low, which is what we observe (Fig. 1b, Fig. 5a, b). However, *High CO_2-cells* of *Chlorella vulgaris* 211-11h tested under 3% CO_2 under our conditions do not excrete glycolate nor do they excrete ammonia in the presence of L-MSO. It can only mean that *Chlorella vulgaris* when compared to *Chlamydomonas* or *Coccochloris* behaves as an exception and succeeds in bringing from the offered 3% CO_2 more to the site of CO_2 fixation than is usual. In this case the CO_2 concentration would be high enough to suppress photorespiration.

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